Clean Specification

A FIBRIL-BLOCKING PEPTIDE, A METHOD FOR PREVENTING FIBRIL FORMATION

BACKGROUND OF THE INVENTION

This Application is based on Provisional Patent Application 60/165/424 filed on November 14, 1999.

1. Field of the Invention

This invention relates to a fibril-preventing peptide and a method for preventing the formation of fibrils and more particularly, this invention relates to a peptide and a method for preventing the formation of solid protein structures and disease associated therewith.

2. Background of the Invention

"Conformational diseases," share a common etiology whereby proteins fold irregularly to produce structural flaws. These flaws result in the proteins unnaturally aggregating and thereafter precipitating as fibrils from their solvents. Such solvents include blood, urine, water, lymph, cerebrospinal fluid, and other physiological fluids. Diseases such as sickle cell anemia, amyloid light chain disease, senile systemic amyloidosis, Alzheimer's, and prion encephalopathies including kuru and "mad cow" disease or BSE, are the result of protein conformation anomalies.

The immunoglobulin light chain (LC) is normally a soluble, secreted protein, but some light

Peptides derived from the VL sequence, including known BiP-binding peptides, were tested for their ability to prevent SMA fibril formation. Eighteen synthetic peptides spanning most of the V_L sequence were tested. Among the eighteen synthetic peptides were FTLEISR (SEQ ID NO: 12) and LTLKLSR (SEQ ID NO: 13). Of them, PKLLIYWA (44-51) exhibited partial inhibition and the overlapping peptides TDFTLTI (amino acids 69-75) and FTLTISS (71-77) markedly inhibited the aggregation. The peptide FTLKISR, a 71-77 sequence common to a number of V_L germline genes inhibited as well as FTLTISS and also inhibited fibril assembly of the peptide $\lambda 6$ V_L protein. Specific structural features of the sequence 71-77 are important for inhibiting aggregation. Peptides in which Phe71 or Ile75 were replaced by Leu were effective inhibitors. However, a peptide with the same amino acids as in the native sequence but in a scrambled order did not inhibit, nor did any of the peptides with Phe71, Leu73 or Ile75 in FTLKISR replaced by Ala. Placement of a Pro in a middle of the peptide also abolished its inhibitory capacity, suggesting that the extended conformation of the peptide is essential.

The inventors have found that the same features of the peptide required for inhibiting aggregation are also necessary for this peptide's binding to BiP. The ability of both BiP and its target V_L peptide to inhibit aggregation therefore suggests that under aggregation-promoting conditions, the loop containing the TDFTLTISS peptide in SMA is not anchored properly in the body of the V domain. The peptide inhibits fibril formation through interactions between its hydrophobic patch on partially unfolded SMA that mimics the BiP binding site.

The readiness with which SMA adopts fibrillogenic conformation as compared to LEN must be due to the minimal sequence differences between the two.

In addition to suggesting a molecular mechanism for light chain amyloidosis, the inventors' results also imply that many somatic mutants of immunoglobulin are involved in such aggregation. Only one (or a few) destabilizing mutation(s) is required to convert a soluble protein to an aggregate prone variation. Further, even if such a mutant does not aggregate spontaneously, it may be induced to form fibrils by the presence of another aggregating light chain, a potentially common *in vivo* event. Identification of these specific structural features should facilitate future development of rational strategies for drug discovery across the spectrum of amyloid disease.

Taken together, these experiments show that the fate of misfolded, amyloidogenic LC was altered by increased interactions with Hsp70 family chaperones on either side of the ER membrane: more SMA was retained in the ER, less was aggregated and more of it remained in a soluble state, whether in the lumen of the ER or in the cytosol. Since the majority of SMA at steady state is in the dislocated, cytosolic pool, BiP would be expected to have less of an effect than Hsp70 when the total cellular protein is analyzed and this is indeed what we observed.

The inventors also found that An Hsp70-binding peptide derived from the LC sequence inhibits SMA aggregation in vivo. To optimize delivery of the peptide to all cellular compartments, it was synthesized with the 11-mer sequence from the HIV TAT protein at the N-terminus (Gius et al., 1999). This TAT peptide permits the transduction of denatured proteins across cell membranes rapidly and efficiently in an energy- and receptor-independent fashion. In addition to the test peptide, TAT-TISS, another TAT-fusion was employed as a specificity control. This peptide, TAT-PASS (SEQ ID NO: 10), contains four amino acid substitutions and does not inhibit fibril formation *in vitro*. SMA transfected cells were incubated overnight in the presence of increasing concentrations of ALLN and 50 μM of each peptide. In the range of 1-10 μg/ml ALLN, there was a progressive increase in the amount of SMA found in the soluble fraction on a per cell basis. Inclusion of the TAT-TISS peptide dramatically reduced the amount of SMA recovered at all ALLN concentrations tested. In contrast, the TAT-PASS (SEQ ID NO: 10) peptide had no effect. Incubation of the same blots with anti-raf antibody demonstrated that equal cell equivalents were loaded across the gel.

The inventors also determined the effect of different concentrations of peptide on SMA following treatment with 10 μg/ml ALLN. The TAT-TISS peptide decreased the yield of SMA in the insoluble fractions much more than in the detergent soluble fractions. The magnitude of the decrease was from 4 to 10-fold (n=3), in a peptide concentration-dependent fashion, whereas the TAT-PASS (SEQ ID NO: 10) peptide had only a marginal effect even at the highest concentration used. As observed with co-expression of Hsp70, upon addition of the TAT-TISS peptide, the ubiquitinated forms of SMA were diminished. This indicated that they were being maintained in a soluble form long enough to be kept off the aggregation pathway and were degraded by the proteasome.

Lastly, the inventors determined that the decrease in steady state level of SMA in the presence

of TAT-TISS peptide correlated with a decrease in the frequency of aggresome formation, by scoring anti-kappa stained cells. Roughly 30% of untreated cells exhibited aggresomes and this number increased to about 65% upon addition of ALLN. Transduction of TAT-TISS decreased the number of aggresomes by more than half, to 25%, about the same as in untreated cells, whereas addition of TAT-PASS (SEQ ID NO: 10) peptide had no significant effect. Hence, the large decrease in insoluble SMA observed in the presence of TAT-TISS peptide coincides with a drop in aggresome formation.

In summary, the inventors have found that peptides which mimic the structure of amyloid-forming proteins are suitable agents in inhibiting fibril formation. Insertion of the peptides into a particular groove typically occupied by an adjacent fibril unit interrupts fibril assembly and thus prevents fibril formation.

When amyloidogenic light chain (LC) fails to fold properly it is dislocated out of the endoplasmic rediculum to the cytosol where two linked coping mechanisms are present. While much of the expelled light chain is degraded by proteasomes, another fraction of the light chain aggregates. The inventors found that the introduction of a specific peptide into the cell inhibits aggregation and increases the efficiency of LC degradation.

The presence of a non-releasing BiP mutant (or high levels of wild type BiP) traps SMA molecules in the ER lumen that would otherwise be disloacted to the cytosol for aggregation.

Alternatively, once dislocated, interaction of SMA with cytosolic Hsp 70 improves the efficiency of aggregation. Over-expression of Hsp 70 decreased the insoluble and ubiquitin-tagged pools of SMA that accumulate in the presence of proteasome inhibitors. In the absence of proteasome inhibitors, however, the level of endogenous Hsp 70 was sufficient to support degradation of most SMA light chains. The inventors have identified a kinetic competition between degradation and aggregation: when there is sufficient Hsp 70 present to keep SMA soluble, more of it gets degraded, but if the level of Hsp 70 is inadequate, then more SMA aggregates. SMA is a type of proteasomal substrate whose degradation is improved by chaperon interactions.

FIG. 4 depicts the inventor's competitive inhibition model. SMA fails to progress along a productive folding pathway and hence both of its domains remain in the reduced state *in vivo*. The presence of the highly unstable κ4 protein is detected by BiP, presumably during or soon after its

translocation across the ER membrane. Binding to BiP prevents SMA aggregation in the lumen and facilitates its dislocation back to the cytosol. Once there, binding to Hsp70 (or related chaperones) serves to maintain SMA in a degradation-competent state, so that it can be ubiquitinated and rapidly targeted to proteasomes. At the same time, Hsp70 inhibits the tendency of SMA to aggregate in the cytosol, thus regulating the balance between degradation and aggregation.

The inability to fold exposes (at least) the two major peptides in each of the two β sheets of the V domain that are good sites for binding of Hsp70 family chaperones. Continued exposure of these sites enables associations first with BiP (within the ER) and then with Hsp70 (in the cytosol). The FTLTISS peptide which is effective in reducing intracellular aggregation has the sequence of one of these two major sites, and importantly, the same features that are required for its anti-aggregation activity are necessary for its Hsp70 binding activity.

The inventors envisage the peptide to interact with the same amino acids in the hydrophobic core of the V domain normally occupied in the folded molecule by the endogenous FTLTISS peptide. In this way, the peptide acts as a surrogate chaperone, inhibiting aggregation and promoting degradation. This provides a new avenue for treatment modalities using rationally designed peptides to suppress aggregation.

While the invention has been described through the embodiments disclosed herein, it should be noted that the embodiments are not intended to limit the scope of the following claims.